tumor suppressor genes during tumorigenesis. Recombination events are not detected by cytogenetic assays. Liechty *et al.* (1998) speculated that the MLA is able to detect recombination because of the autosomal location and the heterozygous status of the *Tk* gene. There is also evidence that the MLA can detect aneuploidy. It is especially important for regulatory purposes to collect more evidence demonstrating that the MLA is capable of detecting aneuploidy and recombination.

For our analysis, we needed mutants that were the result of deletion, mitotic recombination, or aneuploidy. For this purpose, we used three chemicals (AZT, mitomycin C, and taxol) to induce *Tk* mutants. AZT is a thymidine analogue that is clastogenic (Wang *et al.*, 2007). Mitomycin C is a potent clastogen, and it induces mutations in the *Tk* gene (Davies *et al.*, 1993; Dobrovolsky *et al.*, 2002). Taxol can impair cell spindles and induce aneuploidy (Ikui *et al.*, 2005; Mailhes *et al.*, 1999; Schiff and Horwitz, 1980).

As mentioned earlier, Honma *et al.* (2001) performed an analysis of *Tk* mutants using two aneugens, colchicine and vinblastine. The increase in MF was not very high, even after a 24-h treatment (3.6- and 2.3-fold over the control, for colchicine and vinblastine, respectively), so the mutants isolated and analyzed from these chemically treated cultures would include a large number of spontaneous mutants. In the present study, the MFs were much higher (4.8- to 16.6-fold over the control), indicating that many to most of the analyzed mutants were induced by the test chemicals.

Early cytogenetic studies of MLA *Tk* mutants showed that many SC mutants have recognizable chromosome aberrations involving the chromosome 11 that carries the Tk^+ allele. At that time, the chromosome aberrations identified in *Tk* mutants were primarily translocations (Blazak *et al.*, 1986; Hozier *et al.*, 1981; Moore *et al.*, 1985). Later, Southern blot analysis and an allele-specific PCR technique were used to determine the status of the *Tk* allele (Applegate *et al.*, 1990; Liechty *et al.*, 1994, 1996). Most of the SC mutants (both spontaneous and from treated cultures) and a large fraction of LC mutants (depending upon the mutagen) showed the loss of the Tk^+ allele, which indicates that the MLA is able to detect LOH, the most common mutational mechanism in human cancer.

Liechty *et al.* (1998) analyzed a large number of spontaneous *Tk* mutants using LOH analysis as well as chromosome painting. Their analysis provided evidence to support the hypothesis that the MLA detects recombination. However, the mutants they analyzed were all spontaneous

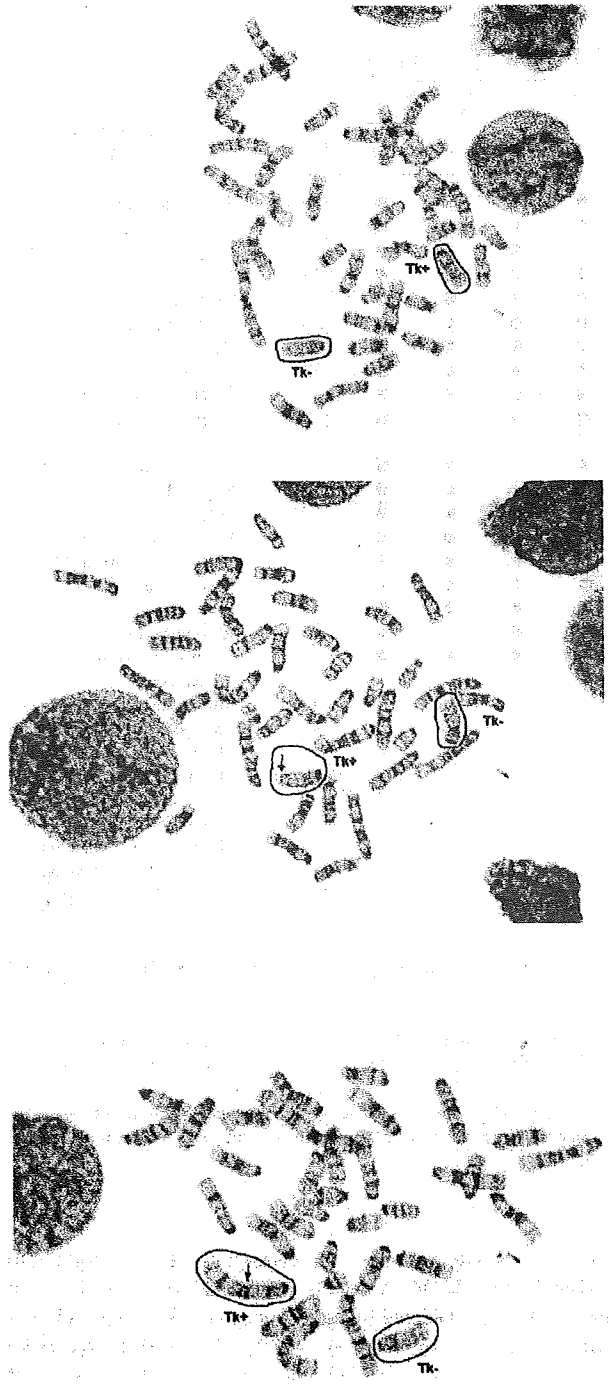

FIG. 2. G-banding analysis of mouse lymphoma *Tk* mutants A3C2, A6C2, and A5D6 that were isolated from a culture treated with 1 mg/ml AZT. The circled chromosomes are chromosome 11. The analysis of mutant A3C2 (top photo) shows a normal metaphase: two chromosome 11 with normal length and banding patterns (Tk^+ chromosome is on the right). The analysis of mutant A6C2 (middle photo) shows a visible deletion of the Tk^+ chromosome (left, indicated by an arrow). The Tk^- chromosome (right) has a normal banding pattern. The analysis of mutant A5D6 (bottom photo) shows a visible deletion of the distal Tk^+ chromosome resulting from an unbalanced translocation (translocation site indicated by an arrow). The Tk^- chromosome (right) has a normal banding pattern. Note that the Tk^+ chromosome has a bigger centromere than the Tk^- chromosome. This centromeric heteromorphism can be used to distinguish between the Tk^+ and Tk^- chromosomes.

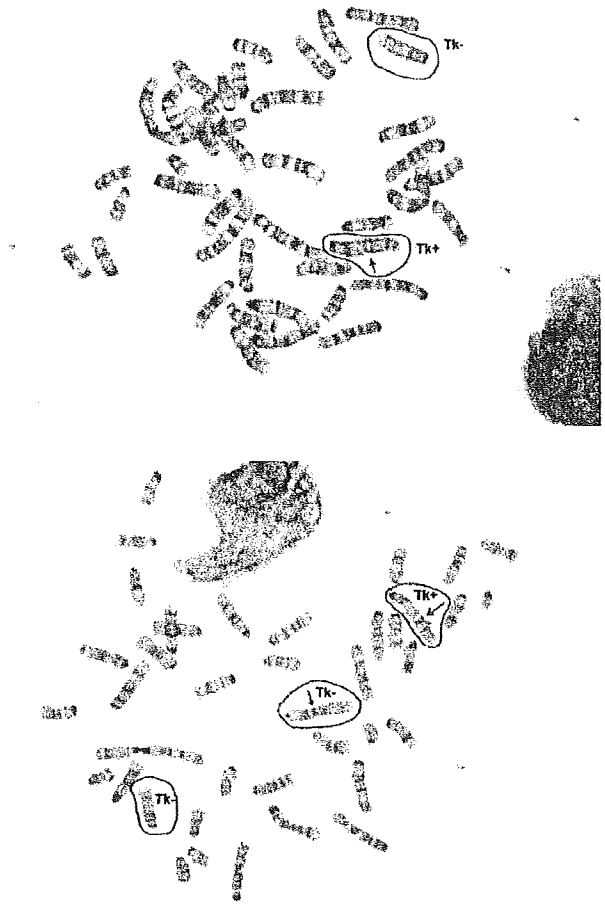

FIG. 3. G-banding analysis of mouse lymphoma *Tk* mutants MS8 (isolated from a cell culture treated with 0.4 μg/ml mitomycin C) and mutant A5D2 (isolated from a cell culture treated with 1 mg/ml AZT). Their metaphase cells display complex chromosome alterations. The circled chromosomes are chromosome 11. The Tk^+ chromosome has a bigger centromere than the Tk^- chromosome. This centromeric heteromorphism can be used to distinguish between the Tk^+ and Tk^- chromosomes. In the top photo for mutant MS8, the Tk^- chromosome (top) shows a normal banding pattern, while the Tk^+ chromosome (bottom) is abnormally long. It is formed by two chromosome 11 joined together (translocation site indicated by an arrow). In the bottom photo for mutant A5D2, two Tk^- chromosomes were identified: one (left) shows a normal banding pattern; the other (middle) is translocated to another chromosome (translocation site indicated by an arrow). The Tk^+ chromosome (right) was partially deleted by an unbalanced translocation (translocation site indicated by an arrow).

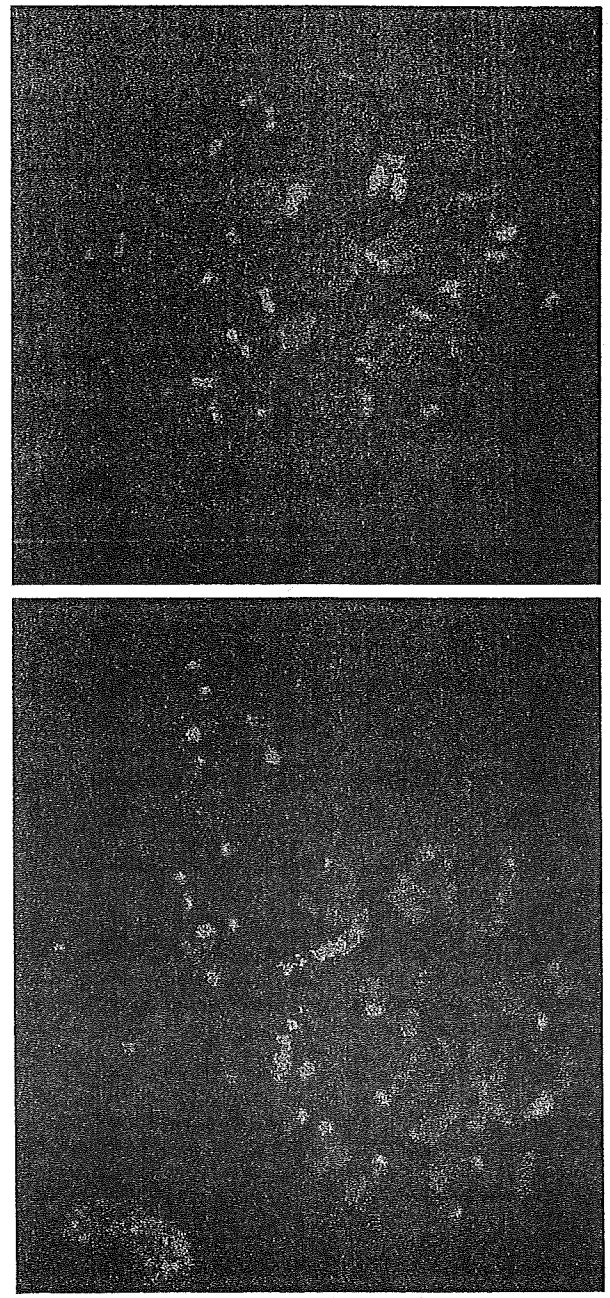

FIG. 4. Chromosome painting analysis of mouse lymphoma *Tk* mutants TL8 and TL2 that were isolated from a culture treated with 1 μg/ml taxol. The chromosome 11 probe is labeled with red fluorescence. The top photo for mutant TL8 shows only one chromosome 11 (chromosome loss). The bottom photo for mutant TL2 shows two chromosome 11 (chromosome duplication after loss).

mutants. In addition, although chromosome painting is relatively easy to perform and analyze, it is not as informative as conventional G-banding analysis. In their study, four mutants showed interesting chromosome alterations: two chromosome 11 with different lengths. Without G-banding analysis, those aberrations could not be identified. Previously we analyzed a mutant isolated from a bleomycin-treated culture (mutant 950, Clark *et al.*, 2004) and chromosome painting showed a similar result: two chromosome 11 with different lengths, with a partial LOH pattern. This was first interpreted as a deletion. However, further analysis using G-banding revealed

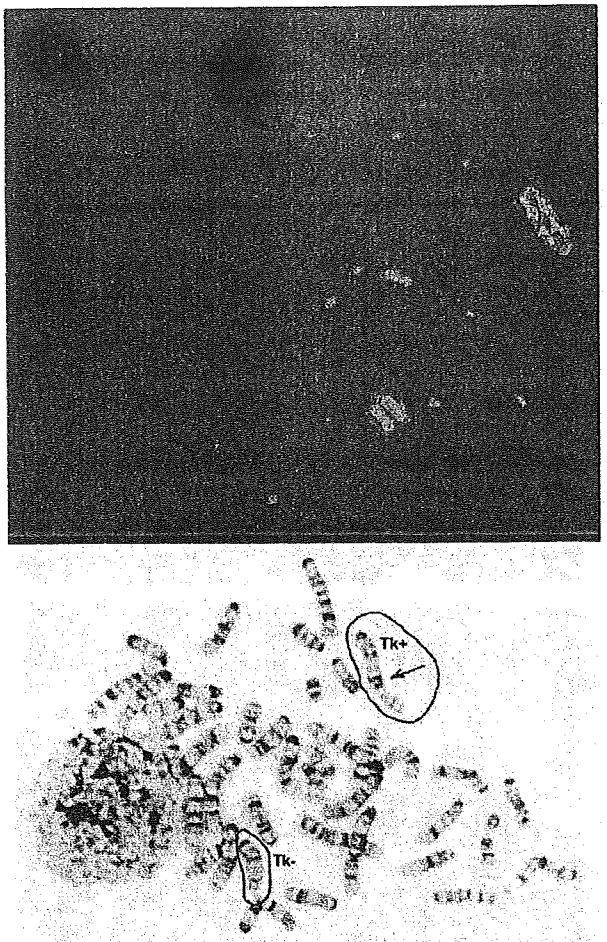

FIG. 5. Chromosome painting and G-banding analysis of mouse lymphoma *Tk* mutant 950 isolated from a bleomycin-treated culture (Clark *et al.*, 2004). The chromosome 11 probe is labeled with red fluorescence. Chromosome painting (top photo) shows two chromosome 11 of different length. The G-banding analysis (bottom photo) shows complex chromosome alterations. The circled chromosomes are chromosome 11. The Tk^- chromosome (left) shows a normal banding pattern, while the Tk^+ chromosome (right) is abnormally long. It is formed by two chromosome 11 joined together (translocation site indicated by an arrow). Note that the Tk^+ chromosome has a bigger centromere than the Tk^- chromosome. This centromeric heteromorphism can be used to distinguish between the Tk^+ and Tk^- chromosomes.

that the "longer" chromosome 11 was actually formed by two (or two parts of) chromosome 11 in an unbalanced translocation, while the "shorter" chromosome 11 was actually a normal Tk^- chromosome (Fig. 5). Therefore, in this study we used a combination of all the different analysis methods to identify the mutation types. Interestingly, five mutants (four from the AZT treatment and one from mitomycin C) were found to have alterations similar to mutant 950: deletion with aneuploidy. The proportion of this specific aberration in the mutants we analyzed is very high: 5 of the 19 analyzed were partial chromosome 11 LOH mutants. The underlying mechanism for this event is unclear. We speculate that the Tk^- chromosome duplication is some type of compensation for the deletion of the Tk^+ chromosome, resulting in partial trisomy (aneuploidy). This unique aberration may be related to the clastogenicity of the chemical: AZT, mitomycin C, and bleomycin are all clastogens.

Although chromosome loss is the primary mutation mechanism whereby aneugens induce Tk^--deficient mutants, few cells were found to be monosomic for chromosome 11 in this study. We analyzed nine mutants showing complete chromosome 11 LOH. The majority were mosaic with most having chromosome duplication after loss. This probably occurs because duplication of the Tk^- chromosome is a repair/compensation mechanism after the loss of the Tk^+ chromosome. Cells containing two Tk^- chromosomes would be expected to have a growth advantage over cells containing only one Tk^- chromosome, thereby becoming the predominant cell type in the culture (Honma *et al.*, 2001).

The results clearly demonstrate that MLA *Tk* mutants can result from recombination, deletion, and aneuploidy. The ability to detect recombination is a particular advantage of the MLA. Recombination is an important pathway for repairing DNA double-strand breaks, and it is essential for cellular survival in mammals (Helleday, 2003). It cannot be detected by assays using hemizygous reporter genes, such as *Hprt*. The ability to detect large deletions is another advantage of the MLA. This may be due to the *Trp53* status of the L5178Y/$Tk^{+/-}$ mouse lymphoma cell line. In this cell line, both alleles of the *Trp53* gene have point mutations, one of which likely results in no protein production (the point mutation produces a stop codon) and the other results in the production of a mutant Trp53 (Clark *et al.*, 1998; Storer *et al.*, 1997). Large-scale damage may be incompatible with the survival of Trp53-sufficient cells; these cells will undergo apoptosis under the surveillance of Trp53 (Honma *et al.*, 2000). The Trp53 status may also play a critical role in the ability of the MLA to detect aneuploidy (Honma *et al.*, 2001). In mammalian cells, Trp53 is involved in the maintenance of diploidy by participating in a mitotic checkpoint and the regulation of centrosome duplication (Cross *et al.*, 1995; Honma *et al.*, 2001; Tarapore and Fukasawa, 2000).

It should be emphasized that while this analysis indicates that the MLA can detect newly induced deletions, mitotic recombination, and aneuploidy, it does not provide insight into the relative proportion of the various types of mutational events or the efficiency with which these events are detected. While we have combined several powerful techniques to elucidate the mutations, all these techniques are very "blunt" tools. The nine LOH markers were distributed across chromosome 11, but from the perspective of potentially mutable sites, only a tiny fraction of the chromosome could be evaluated. Furthermore, our strategy of combining the G-banding analysis with the LOH analysis to distinguish between deletion and mitotic recombination required that the breakpoints be located so that at least approximately 25%

of the chromosome would be deleted (and readily visible by banded karyotype) if the mutant were a deletion rather than resulting from mitotic recombination.

To understand the mechanism for the induction of every mutant, and ultimately to fully understand the fundamental differences between the small and LC *Tk* mutants, the analysis must be conducted in a way that can interrogate a much larger portion of chromosome 11. We are currently initiating a research project to utilize comparative genomic hybridization microarray technology in combination with our current tools.

Our present study clearly demonstrates that the MLA can, in fact, detect deletion, recombination, and aneuploidy and provides new evidence for the utility of the MLA in a mechanistically based genotoxicity hazard identification battery. Depending upon the question that is being addressed and the importance of understanding the mutations induced by a particular chemical, our strategy of combining cytogenetic and molecular analysis of mutants can be used to provide more than a simple mutagenic/nonmutagenic hazard assessment.

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An Approach to Estimate Radioadaptation from DSB Repair Efficiency#

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Radioadaptation/I-SceI digestion/DSB repair/TK6 cells.

In this review, we would like to introduce a unique approach for the estimation of radioadaptation. Recently, we proposed a new methodology for evaluating the repair efficiency of DNA double-strand breaks (DSB) using a model system. The model system can trace the fate of a single DSB, which is introduced within intron 4 of the *TK* gene on chromosome 17 in human lymphoblastoid TK6 cells by the expression of restriction enzyme I-SceI. This methodology was first applied to examine whether repair of the DSB (at the I-SceI site) can be influenced by low-dose, low-dose rate gamma-ray irradiation. We found that such low-dose IR exposure could enhance the activity of DSB repair through homologous recombination (HR). HR activity was also enhanced due to the pre-IR irradiation under the established conditions for radioadaptation (50 mGy X-ray–6 h–I-SceI treatment). Therefore, radioadaptation might account for the reduced frequency of homozygous loss of heterozygosity (LOH) events observed in our previous experiment (50 mGy X-ray–6 h–2 Gy X-ray). We suggest that the present evaluation of DSB repair using this I-SceI system, may contribute to our overall understanding of radioadaptation.

INTRODUCTION

It is important to accurately estimate human health risks for persons occupationally exposed to ionizing radiation (IR), such as airline crews and workers in medical and industrial fields. For estimating such risks, it is worthwhile to investigate radioadaptation, that is, acquiring a cellular radioresistance to a challenging IR by a pre-exposure to low-dose IR. Radioadaptation was first reported by Olivieri *et al.*[1] The priming radiation exposure delivered by labeling human lymphocytes with tritiated thymidine caused a decrease in chromosomal aberration frequency after a challenging exposure to 1.5 Gy of IR. That discovery stimulated a series of studies using human lymphocytes and various mammalian cell lines as described in reviews.[2,3] A reduced induction of both micronuclei and sister chromatid exchanges was shown in Chinese hamster V79 cells pre-exposed to low doses of γ-rays or ^{3}H β-rays.[4] Subsequent studies reported similar radioadaptive responses, such as reduced mutation frequencies in human lymphocytes,[5] mouse SR-1 cells[6] and human-hamster hybrid A_L cells,[7] an altered mutation spectrum in human-hamster hybrid A_L cells,[7] reduced micronucleus frequencies in human lymphocytes[8] and mouse embryo cells,[9] and reduced deletions and rearrangements in human lymphoblast cells.[10] Those studies suggest that radioadaptation is an important defense mechanism against a high-dose IR, although the molecular mechanisms involved remain largely unknown.[11–15]

Cellular responses such as a bystander effect, genetic instability, and hyper-radiosensitivity are reported to be tightly related to the radioadaptation.[16–21] In mammalian cells, for example, bystander mutagenesis may be suppressed by an adaptive response.[16] Another example is the possible involvement of a "radioadaptive bystander" effect in human lung fibroblasts.[22] The reduction of radiosensitivity in cells with a wild type *p53* gene by a radiation-induced, nitric oxide (NO)-mediated bystander effect may also be a manifestation of the radioadaptation.[21,23] This possibility is supported by the finding that the NO-induced apoptosis observed in lymphoblastoid and fibroblast cells depends on the phosphorylation and activation of p53.[24] In fact, p53 was suggested to play a key role in the mechanisms of an adaptive response

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mediated by a feedback signaling pathway involving protein kinase C (PKC), p38 mitogen activated protein kinase (p38MAPK), and phospholipase C (PLC).[11,13,25]

One of the possible targets for radioadaptation is oxidative base damage. A low-dose rate whole body γ-irradiation of mice (1.2 mGy/h, total 0.5 Gy) demonstrated the activation of antioxidative enzymes such as MnSOD and catalase in spleen cells, leading to less DNA damage as determined by a comet assay.[26] Furthermore, down-regulation of the human *CDC16* gene that occurs after oxidative stress causes more rapid and efficient repair in adapted (2 cGy pre-irradiated) human lymphoblastoid cells challenged with 4 Gy irradiation.[12] However, oxidative base excision repair enzymes, including DNA glycosylases, hOGG1, and hNth1, are reportedly not up-regulated at the post-transcriptional level in γ-ray-primed TK6 cells.[27] Those reports suggest that the antioxidant defense machinery is likely to be involved in radioadaptation although the mechanisms involved are still not well understood.

Gene expression also seems to be tightly related to a variety of functions in the adaptive response such as the induction of antioxidant defense machinery, repair of DNA damage, control of cell-cycle progression, *etc.* In fact, *de novo* synthesis of transcripts and proteins is reported to be required for the expression of the adaptive response.[28] Following that report, gene expression analysis has been extensively studied by many investigators.[15,28–31] For example, the *CHD6* gene in human lymphoblastoid cell AHH-1 can be up-regulated by 0.5 Gy of γ-irradiation and its induced expression could be involved in a low-dose hypersensitive response.[29] Recently, gene profiles in the kidney and testis from γ-irradiated (485 days at dose rates of 0.032–13 μGy/min) mice were determined using oligonucleotide microarrays, and differentially expressed genes were identified.[31]

DNA double strand breaks (DSBs) are a most serious type of DNA damage. They can be caused by IR or radiomimetic chemicals, and they can occur spontaneously during DNA replication. The nonrepair or misrepair of DSBs can cause cell death or mutagenic and/or carcinogenic consequences, so the accurate repair of DSBs is important for maintaining genomic integrity.[32,33] In other words, DSB repair is an essential function in all living organisms. Recently analyses using nondividing lymphocyte and fibroblast cells suggested that the adaptive response is not mediated by an enhanced rejoining of DNA strand breaks but rather is a reflection of perturbation in cell cycle progression.[34] On the other hand, the induction of an efficient chromosome repair system by the priming radiation dose is considered to be involved in radioadaptation mechanisms, and in fact, the efficiency of DSB repair in Chinese hamster V79 cells exposed to γ-rays is enhanced by a priming exposure of 5 cGy of γ-rays.[35] The reduced frequencies of chromosomal alterations as described above supports the latter possibility of DSB-repair enhancement. At the present stage, it is difficult to conclude which factor, cell-cycle perturbation or DSB repair, largely contributes to radioadaptation.

THE I-SCEI SYSTEM FOR DSB REPAIR EVALUATION

Outline of the system

A model system was constructed for evaluating DSB repair by tracing the fate of a single DSB on chromosomal DNA. The DSB generated in this system can be considered as a target DNA-lesion susceptible to repair, and this system can distinguish two major DSB repair pathways, non-homologous end-joining (NHEJ) and homologous recombination (HR) (Fig. 1).[36,37] The human lymphoblastoid cell line TSCE5 is heterozygous (+/–) for the thymidine kinase (*TK*) gene and the line TSCER2 is compound heterozygous (–/–; two different TK^- alleles); both carry an I-SceI endonuclease recognition site in intron 4 on one allele of the *TK* gene. DSBs can be generated at the I-SceI site by expression of the I-SceI vector.[36,37] When DSBs occur at the *TK* locus, NHEJ in TSCE5 cells produces TK-deficient mutants while HR between the *TK* alleles in TSCER2 cells produces TK-proficient revertants. This means that positive-negative drug selection for TK phenotypes permits distinction between NHEJ and HR repair.

Cell line construction for use in the system

Details of the strain construction are described in our previous work (Fig. 2).[36] Briefly, in lymphoblastoid TK6 cells heterozygous for the *TK* gene, the functional allele was first inactivated by gene targeting with vector pTK4 to

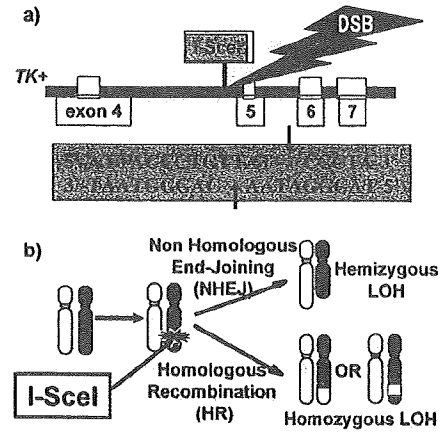

Fig. 1. Principle of DSB formation and repair evaluation. A single DNA double strand break (DSB) is generated at the I-SceI recognition site in a human lymphoblastoid TK6 cell by transfecting an I-SceI expression vector (a) and the efficiencies of DSB repair through non-homologous end-joining (NHEJ) or homologous repair (HR) are evaluated from induction of hemizygous and homozygous LOH events, respectively (see text).

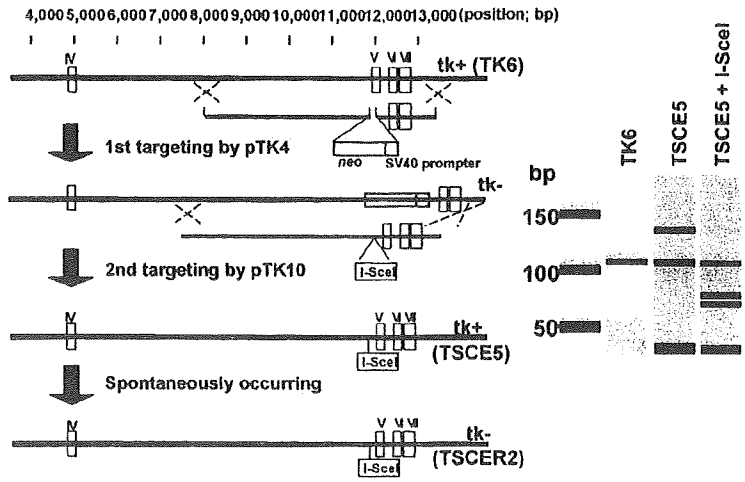

Fig. 2. Cell line construction. In the TK6 cell line, the functional allele of *TK* gene was first inactivated by gene targeting vector pTK4 and then the I-SceI recognition site was introduced at 75 bp upstream of exon 5 in the *TK* gene. The new line was termed TSCE5 and its compound heterozygote (*TK*–/–) cell line, TSCER2, was also isolated (see text).

replace exon 5 of the *TK* gene by a *neo* gene. To introduce the I-SceI recognition site at 75 bp upstream of exon 5, the targeting vector pTK10, encompassing about 6 kb of the original *TK* gene with exons 5, 6, and 7, and the I-SceI recognition site in intron 4, was used to revert the *TK* gene disrupted by pTK4. The new line was termed TSCE5. A spontaneous mutation in a TSCE5 cell (G to A in position 23 of exon 5), which we cloned, led to the compound heterozygote (*TK*–/–) cell line, TSCER2.

I-SceI expression for introduction of DSB

We introduced the I-SceI expression vector (pCBASce) by electroporation methodology using Nucleofector Kit V (amaxa AG, Cologne, Germany) (Fig. 3).[36–38] The I-SceI expression vector was introduced into about 65% of the cells at 24 hr after the transfection and the expression last for 3 days incubation.[37] The relatively long expression allowed us to succeed in estimating the influence of low-dose, low-dose-rate γ-rays irradiation on DSB repair, especially the effect of post-IR-exposure, as described below.

Evaluation of DSB repair efficiencies

Measurements of TK^- mutants and TK^+ revertants allow us to evaluate DSB repair efficiencies through NHEJ and HR pathways, respectively (Fig. 3). In TSCE5, when a DSB at the I-SceI site is repaired by NHEJ involving a deletion in the adjacent exon, the cell can be isolated as a TK-deficient mutant. In TSCER2, when a DSB is repaired by HR between the *TK* alleles, a TK^+ allele can be generated, resulting in a revertant phenotype. The DSB repair *via* NHEJ was 73–86 times higher than that *via* HR in our previous studies.[36,37] These findings are consistent with the report that NHEJ is the major repair pathway in mammalian cells.[39]

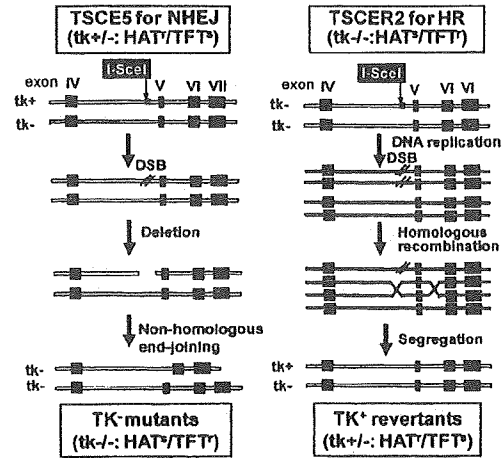

Fig. 3. An approach to evaluate DSB repair efficiency. In TSCE5, when a DSB at the I-SceI site is repaired by NHEJ involving a deletion in the adjacent exon, the cell can be isolated as a TK-deficient mutant. In TSCER2, when a DSB is repaired by HR between the *TK* alleles, a TK^+ allele can be generated, resulting in a revertant phenotype (see text). Filled exons represent TK mutations.

APPLICATION OF THE I-SCEI SYSTEM FOR EVALUATING RADIOADAPTATION IN TERMS OF DSB REPAIR

Influence of low-dose, low-dose-rate γ-rays on DSB repair

The I-SceI digestion system was applied for estimating the influence of low-dose, low-dose-rate γ-irradiation on repair of a site-specifically introduced DSB (Fig. 4).[38] The results

obtained with Mode A (30 mGy of pre-γ-irradiation) and Mode B (8.5 mGy of post-γ-irradiation) are shown in Tables 1 and 2, respectively. The NHEJ repair of DSB was little influenced by either modes of low-dose, low dose-rate γ-irradiation. DSB repair by HR, in contrast, was enhanced by ~50% and ~80% in Mode A and Mode B, respectively. This might implicate that both pre-γ-irradiation (Mode A) and post-γ-irradiation (Mode B) induce a radioadaptation, although both modes of irradiations, especially Mode B, are different from the original concept of radioadaptation. In fact, DSBs are generated during the γ-irradiation in Mode B, because I-SceI expression lasts for 3 days incubation as previously mentioned.

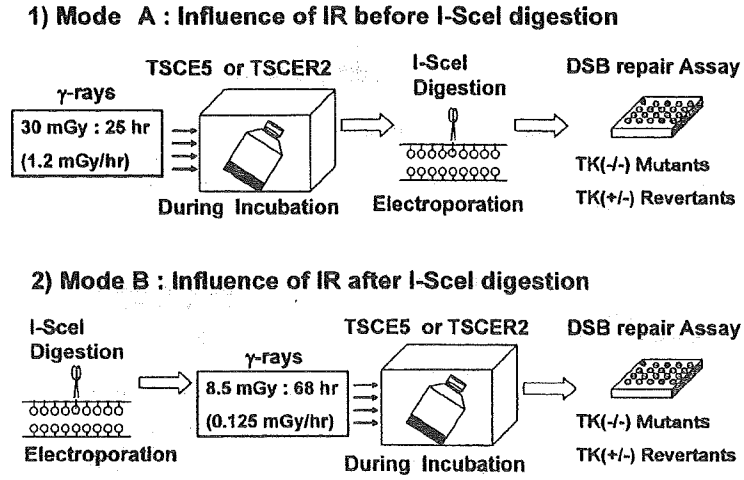

Fig. 4. Influence of low-dose IR exposure on DSB repair. Experimental schemes of radiation exposure and I-SceI expression are illustrated. Mode A: cells were exposed to low-dose, low-dose-rate γ-rays and then transfected with the I-SceI vector by electroporation (see text). 2) Mode B: cells were transfected with the I-SceI vector and then exposed to γ-rays at a much lower dose and dose-rate (see text).

Table 1. Effect of pre-IR exposure on DSB repair (Mode A).

a) NHEJ efficiency in TSCE5 cells

Exp.	Mutant Frequency, MF ($\times 10^{-6}$)				Effect of IR (Relative MF*)
	Control	γ-rays	I-SceI	γ-rays + I-SceI	
1	3.5	6.1	8600	8500	0.99
2	1.8	3.2	2900	3200	1.1
Average	2.7	4.7	5800	5900	1.0 (P = 0.82)

*Relative MF was calculated as MF (γ-rays + I-SceI)/MF (I-SceI).

b) HR efficiency in TSCER2 cells

Exp.	Revertant Frequency, RF ($\times 10^{-6}$)				Effect of IR (Relative RF*)
	Control	γ-rays	I-SceI	γ-rays + I-SceI	
1	–	–	90	114	1.3
2	–	–	62	96	1.5
3	–	–	25	45	1.8
Average	–	–	59	85	1.5 (P = 0.021)

*Relative RF was calculated as RF (γ-rays + I-SceI)/RF (I-SceI).

Table 2. Effect of post-IR exposure on DSB repair (Mode B).

a) NHEJ efficiency in TSCE5 cells

Exp.	Mutant Frequency, MF ($\times 10^{-6}$)				Effect of IR (Relative MF*)
	Control	γ-rays	I-SceI	γ-rays + I-SceI	
1	2.8	1.3	3400	4500	1.3
2	3.1	2.8	12000	17000	1.4
3	–	–	11000	11000	1.0
Average	3.0	2.1	8800	10800	1.2 (P = 0.12)

*Relative MF was calculated as MF (γ-rays + I-SceI)/MF (I-SceI).

b) HR efficiency in TSCER2 cells

Exp.	Revertant Frequency, RF ($\times 10^{-6}$)				Effect of IR (Relative RF*)
	Control	γ-rays	I-SceI	γ-rays + I-SceI	
1	–	–	82	160	2.0
2	–	–	160	270	1.7
3	–	–	110	190	1.7
Average	–	–	120	210	1.8 (P = 0.0013)

*Relative RF was calculated as RF (γ-rays + I-SceI)/RF (I-SceI).

Influence of low-dose X-ray irradiation on DSB repair

We have extensively studied the effects of low-dose IR by using a loss of heterozygosity (LOH) analysis system.[40–42] The thymidine kinase deficient (TK^-) mutants induced in TK6 cells can be classified as LOH type and non-LOH type by this system. The LOH mutants were further classified as homozygous-type and hemizygous-type, and the replaced or deleted part of the chromosome was identified by so-called chromosome mapping. In addition to this kind of analysis at the chromosome level, non-LOH mutants were further characterized at the DNA sequence level to confirm that the mutation occurs in the *TK* gene or not. Recently we could establish the optimum condition for mutagenic radioadaptation in TK6 cells.[43] Under such condition as shown in Fig. 5, the greatest reduction in *TK* mutation frequency was observed in TK6 cells exposed to a challenging X-ray irradiation (2 Gy), and the TK^- mutants so obtained were analyzed by the LOH system.[43]

The TK^- mutation frequency (MF) obtained after the challenging X-ray (2 Gy) exposure, 18.3×10^{-6} was reduced to 11.4×10^{-6} (62% of the original level) by inducing the radioadaptation (50 mGy of pre-X-irradiation at 6 hr before the above challenging X-irradiation; Fig. 6). LOH analysis could classify the TK^- mutational events as non-LOH (mostly mutations in the *TK* gene), hemizygous LOH (deletion of chromosome) and homozygous LOH (homologous recombination [HR] between chromosomes), as mentioned above.[40–42] Non-LOH events are, in theory, classified as chromosomal alterations, but most of non-LOH mutants obtained in this experiment were confirmed to be small mutations in the *TK* gene by DNA base sequencing of mRNA obtained from the mutants.[43] The pre-irradiation decreased the frequencies of non-LOH events and homozygous LOH events to 27% and 60% of the original levels, respectively. The frequency of hemizygous LOH events, however, was not significantly altered by the pre-irradiation. Since LOH events are most likely the consequence of DSB repair, we tried to investigate the influence of priming X-ray irradiation on DSB repair efficiency under the optimum condition for radioadaptation.

The repair efficiency of DSB *via* NHEJ was hardly influenced by the pre-irradiation of 50 mGy X-rays (Table 3). On the other hand, a ~70% enhancement in HR repair of DSB was observed after this treatment. The enhanced activity of HR observed in this experiment could reflect the activity of error-free DSB repair, providing a reduction in genetic alterations at the chromosome level. In fact, we observed a ~60% reduction in the induction of homozygous LOH as mentioned above. The chromosome-mapping analysis demon-

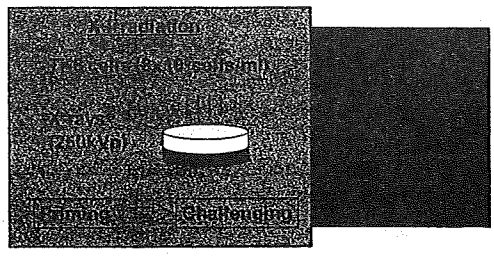

Fig. 5. An experimental scheme for mutagenic radioadaptation. The optimum conditions providing the greatest reduction in the frequency of *TK* mutations induced after a challenging X-ray (2 Gy) irradiation of TK6 cells, are shown in the right panel of this figure. The details have already been described in our previous work.[43]

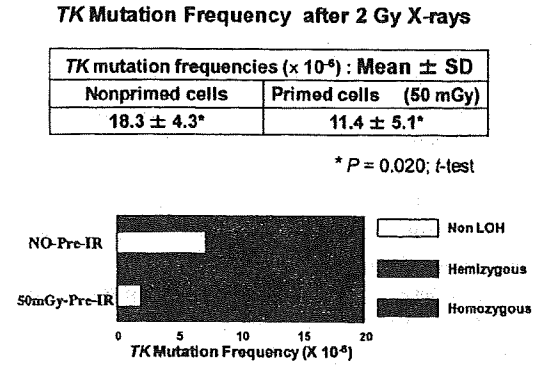

Fig. 6. Genetic analysis of radioadaptation induced by low-dose X-rays. Results of the *TK* mutation assay performed under the optimum condition for radioadaptation (Fig. 5) are summarized in the table, and the classification of the isolated TK^- mutants was made by LOH analysis and the results are shown in the histograms (see text).

Table 3. The effect of a priming X-ray exposure on DSB repair (X-ray - X-ray adaptive experiment).

a) NHEJ efficiency in TSCE5 cells

Exp.	Effect of IR (Relative MF*)
1	0.98
2	0.76
3	0.99
Average	0.91

*Relative MF was calculated as MF (X-rays + I-SceI)/MF (I-SceI).

b) HR efficiency in TSCER2 cells

Exp.	Effect of IR (Relative RF*)
1	2.2
2	1.2
3	1.7
Average	1.7

*Relative RF was calculated as RF (X-rays + I-SceI)/RF (I-SceI).

strated that the observed homozygous LOH events were mostly of the crossing-over type.[23] In contrast, the analysis of TK (+/–) revertants observed with our DSB repair assay suggests that HR in this I-SceI system mostly reflects a gene conversion activity, with a relatively small proportion of non-crossing-over events (data not shown). More supporting evidence is required to determine if an enhanced HR activity is reflected by the reduction in homozygous LOH events.

Further applications and perspectives

It is of theoretical and practical importance to estimate human health risks from low-doses of ionizing radiation. One example is the risk for astronauts exposed to space radiation, because the background radiation in space is, at least, more than 100-fold higher than the background level found on earth. Currently, we have the opportunity to study the influence of space radiation in TK6 cells, which were recently brought back to earth after preservation for more than four months, mostly in a frozen state, in the International Space Station. Assuming that the DNA damage caused by space radiation has been accumulated in the frozen cells, such damage could induce mutations when the cells begin to grow again. Furthermore, such damage might have the potential ability to induce radioadaptation and this radioadaptation might be detected as an enhancement in DSB repair in the I-SceI digestion system in the recovered cells.

The following points involved in our I-SceI digestion system merit discussion. Because our I-SceI system does not uncover all NHEJ and HR events, it is difficult to evaluate accurately the extent of DSB repair *via* both HR and NHEJ pathways. For example, our system does not monitor sister-chromatid HR, which is probably the major HR pathway in mammalian cells. Small gene conversion events, which do not extend into the exon 5 region, can also not be detected by this system. Although the I-SceI system may over-estimate the repair efficiency of NHEJ compared with HR, this methodology can still be considered to contribute to elucidating the DSB repair associated with low-dose IR exposure.

Finally, we would like to emphasize that the present evaluation of DSB repair using the I-SceI system, may contribute to our overall understanding of radioadaptation. Other types of studies regarding gene expression, epigenetic changes *etc.*, are also required for a more complete understanding.

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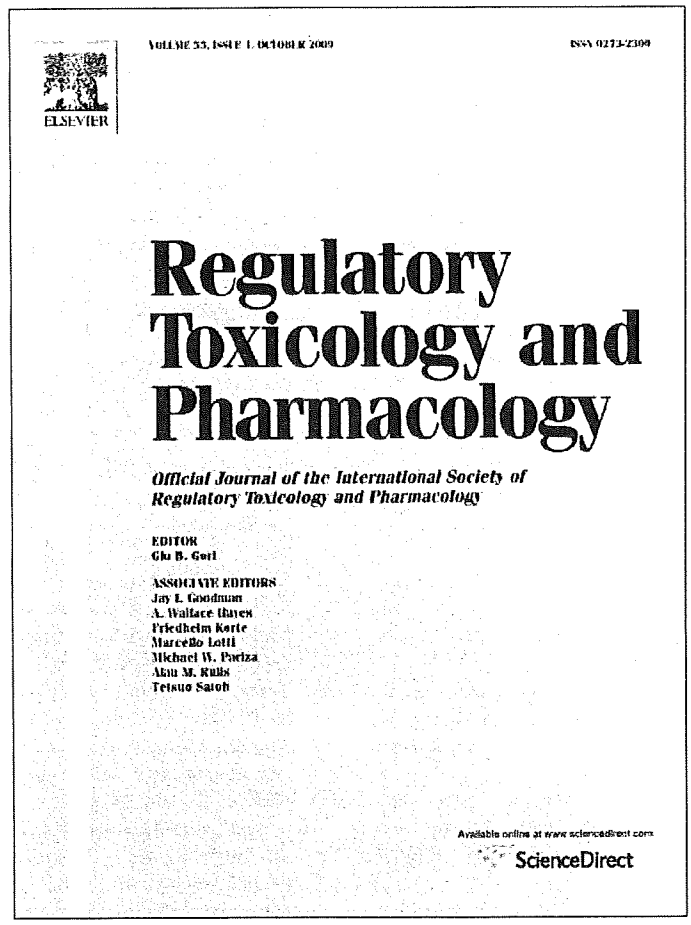

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Practical issues on the application of the GHS classification criteria for germ cell mutagens

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ABSTRACT

The Globally Harmonized System of Classification and Labeling of Chemicals (GHS) requires classification of chemicals on germ cell mutagenicity. The Japanese government has conducted GHS classification on about 1400 chemicals in a 2-year project (J-GHS) for implementing GHS domestically. Prior to the classification work, the technical guidance for classification of germ cell mutagens was prepared. This guidance introduces the concept of heritable mutagenicity, and presents detailed criteria for germ cell mutagens, test data to be used, and a practical decision tree for classification. These practical guidance and supporting explanations are useful for non-expert Classifiers (scientists applying the classification criteria). Several issues, however, were identified during the course of J-GHS and in re-evaluating the classification results. These include: (1) the information sources when available data are limited; (2) lack of understanding GHS classification criteria or insufficient review of the information by Classifiers; (3) varying opinions of experts on data quality and weight of evidence, and; (4) decision tree approaches, e.g., inadequacy for use in overall evaluation in some cases. Ideally, classification should be performed by Classifiers with high expertise using high quality information sources. Genetic toxicologists as experts should consider data quality and reliability, and give a critical review of all available information for support of classification. A weight of evidence approach is also required to assess mutagenic potential of chemicals. Critical points for suitable classification for GHS are discussed.

1. Introduction

Communicating the hazards of dangerous chemicals to workers and the public is a key foundation for protecting human health and the environment. As a major break-through in this area, the Globally Harmonized System of Classification and Labeling of Chemicals (GHS) was adopted by the United Nations Economic and Social Council Subcommittee (UN ECOSOC) of Experts on the GHS in December 2002 and endorsed by ECOSOC in July 2003 (UN, 2003, 2005, 2007). The GHS has the ultimate goal of ensuring that information on chemical hazards (such as on labels and safety data sheets) is made available to workers and consumers in a harmonized and comprehensible format in all countries around the world. The GHS has become the major international tool for effective chemical classification and hazard communication. It represents an important step in harmonizing national chemical hazard communication systems worldwide and has a great potential to improve chemical safety across all relevant sectors. The GHS is a consistent and coherent approach to identifying the hazards of chemicals, and providing information on these hazards and associated protective measures to users or those who may be exposed. The system is structured so that appropriate elements for classification and communication, which consider the target population, can be selected. Those who then use chemicals can take the proper steps to protect themselves and the environment. Target populations include employers, workers (including those involved in transport), consumers, and emergency responders. Others who provide services to these people will also find the information useful (e.g., doctors, toxicologists, nurses, safety engineers and occupational hygienists) (UNITAR, 2007). The GHS covers all hazardous chemical substances, dilute solutions and mixtures. It also addresses how labels and safety data sheets should be used to convey information about their hazards, and how to protect people from these effects. However, pharmaceuticals, food additives, cosmetics, and pesticide residues in food will not necessarily be

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covered by the GHS in terms of labeling at the point of intentional intake.

In response to the recommendation made by the United Nations and the agreement endorsed at an Asia–Pacific Economic Cooperation (APEC) meeting, the Japanese Government has started a series of national projects for the implementation of the GHS. One of the projects is classification of approximately 1400 chemicals which are regulated under current legislation. In Japan, material safety data sheets (MSDS) are required for approximately 1400 chemicals that are regulated by the Industrial Safety and Health Law, the Poisonous and Deleterious Substances Control Law or the Pollutant Release and Transfer Register (PRTR). The Japanese government decided to implement classification of these chemicals as 2-year project (Japanese GHS Classification Project, J-GHS) in 2005 and 2006, aiming to help industries issuing MSDS and to develop infrastructure for GHS implementation. The classification work was performed by experts from laboratories and industries and the results were checked by the members of the Inter-ministerial Committee if deemed necessary. The classification results are not compulsory and allow industries to use their own data and classify chemicals on the basis of their own judgment. The results are available in the websites of National Institute of Technology and Evaluation (NITE, http://www.safe.nite.go.jp/english/ghs_index.html#results).

Prior to the classification work, a "GHS Classification Manual" and a "Technical Guidance Document on the GHS Classification" were developed to facilitate the classification of chemicals within the limited time schedule, and to eliminate any conflicting results amongst experts (both are available in English from the website http://www.safe.nite.go.jp/english/ghs_index.html) (NITE, 2005a, 2005b). The Manual provides the main rules and reliable data sources for classification on physical, health and environmental hazards, e.g., peer-reviewed documents prepared by international authorities or governments. In general original scientific literature was not used. This enabled industries to avoid checking the peer-review of the international organizations in their voluntary classification. The technical guidance provides precise rules for GHS classification in J-GHS on each item of health hazards.

Germ cell mutagenicity is included as one of health hazards in the GHS. Definitions of mutagenicity and genotoxicity, classification criteria for substances or mixtures, and decision logic are provided in the GHS text (UN, 2007). However, these may still create some difficulties for non-experts to understand the definitions and explanations. Therefore, precise descriptions were provided on the Guidance for the classification of germ cell mutagens (GCM). It introduces the concept of heritable mutagenicity, and presents detailed criteria of GCM, mutagenicity or genotoxicity test data to be used, and a flow chart for classification.

GHS classifications were performed in accordance with the Manual and the Guidance in J-GHS. Despite these rules, some inappropriate classifications were found. Several issues for a practical approach to the GCM classification were identified during the review process of the project. In this paper, practical approaches of the classification of GCM and examples are presented and its usefulness is discussed. The issues identified in the classifications are also discussed.

2. GHS classification systems for germ cell mutagens

Category 1 is used for chemicals known to induce heritable mutations (Category 1A) or known to be regarded as if they induce heritable mutations (Category 1B) in germ cells of humans. Category 2 is used for chemicals which cause concern for humans owing to the possibility that they may induce heritable mutations in the germ cells of humans. In the case of no concern of induction of heritable mutations in the germ cells of humans or no sufficient evidence of inclusion in Category 1 or 2, the substances are regarded as "not classified". If there are not enough data to be evaluated to determine the mutagenicity of the substance, it is regarded as "classification not possible" (Fig. 1) (UN, 2007). Hazard categories and the criteria for germ cell mutagens in GHS are summarized in Table 1.

The basic concept of classification criteria in GHS is hazard identification and not risk based evaluation (UN, 2007). The germ cell mutagens should be classified by considering the weight of evidence. For classification, test results are considered from experiments determining mutagenic and/or genotoxic effects in germ and/or somatic cells of exposed animals. Mutagenic and/or genotoxic effects determined in *in vitro* tests may also be considered on a case-by-case basis. The tests should be well conducted and sufficiently validated, preferably as described in OECD Test Guidelines. Evaluation of the test results should be done using expert judgment and all the available evidence should be weighed for

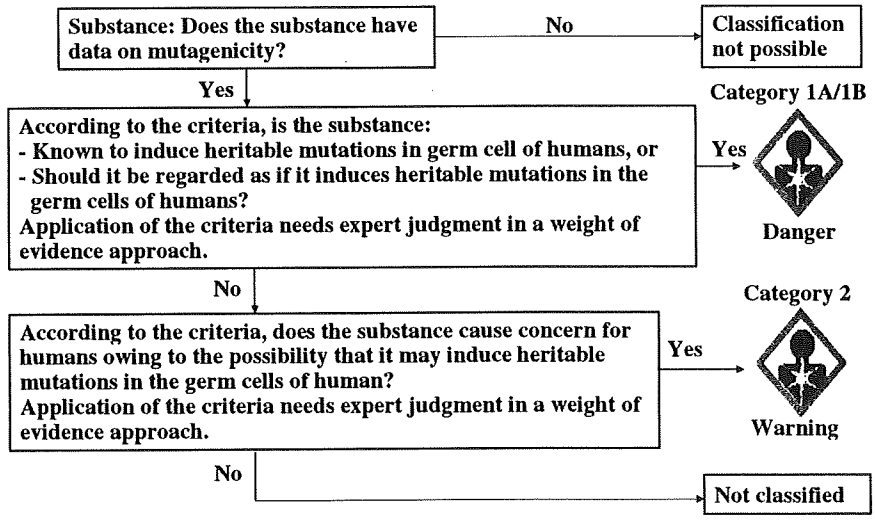

Fig. 1. Decision logic for germ cell mutagenicity for substances in the GHS. The mixture will be classified as a mutagen when at least one ingredient has been classified as a Category 1 or Category 2 mutagen and is present at or above the cut-off value/concentration limit (i.e., 0.1% for Category 1 mutagen, 1.0% for Category 2 mutagen) for Category 1 or 2, respectively.

Table 1
GHS classification on germ cell mutagens.

Category	Classification	Criteria
Category 1	Chemicals known to induce heritable mutations (Category 1A) or to be regarded as if they induce heritable mutations in the germ cells of humans (Category 1B)	
Category 1A	Chemicals known to induce heritable mutations in germ cells of humans	Positive evidence from human epidemiological studies.
Category 1B	Chemicals which should be regarded as if they induce heritable mutations in the germ cells of humans	– Positive result(s) from *in vivo* heritable germ cell mutagenicity tests in mammals; or – Positive result(s) from *in vivo* somatic cell mutagenicity tests in mammals, in combination with some evidence that the substance has potential to cause mutations to germ cells. This supporting evidence may, for example, be derived from mutagenicity/genotoxic tests in germ cells *in vivo*, or by demonstrating the ability of the substance or its metabolite(s) to interact with the genetic material of germ cells; or – Positive results from tests showing mutagenic effects in the germ cells of humans, without demonstration of transmission to progeny; for example, an increase in the frequency of aneuploidy in sperm cells of exposed people. Examples of *in vivo* heritable germ cell mutagenicity tests are: – Rodent dominant lethal mutation test – Mouse heritable translocation assay – Mouse specific locus test Examples of *in vivo* somatic cell mutagenicity test are: – Mammalian bone marrow chromosome aberration test – Mouse spot test – Mammalian erythrocyte micronucleus test Examples of mutagenicity/genotoxicity tests in germ cells are: (a) Mutagenicity tests: – Mammalian spermatogonial chromosome aberration test – Spermatid micronucleus assay (b) Genotoxicity tests: – Sister chromatid exchange analysis in spermatogonia – Unscheduled DNA synthesis test (UDS) in testicular cells
Category 2	Chemicals which cause concern for humans owing to the possibility that they may induce heritable mutations in the germ cells of humans	– Positive evidence obtained from experiments in mammals and/or in some cases from *in vitro* experiments, obtained from: – Somatic cell mutagenicity tests *in vivo*, in mammals; or – Other *in vivo* somatic cell genotoxicity tests which are supported by positive results from *in vitro* mutagenicity assays. Examples of genotoxicity tests in somatic cells are: – Liver UDS *in vivo* – Mammalian bone marrow sister chromatid exchanges Examples of *in vitro* mutagenicity tests are: – *In vitro* mammalian chromosome aberration test – *In vitro* mammalian cell gene mutation test – Bacterial reverse mutation tests

Note. Chemicals which are positive in *in vitro* mammalian mutagenicity assays, and which also show chemical structure activity relationship to known germ cell mutagens, should be considered for classification as Category 2 mutagens.

classification. A single well-conducted test can be used for classification, if it provides a clear and unambiguously positive result. If new and well validated tests are developed, these may also be used in the total weight of evidence. The relevance of the route of exposure used in the *in vivo* study should also be taken into account the comparison with the route of human exposure.

3. Information sources used in J-GHS

It is not only inefficient but also not practical for general industrial Classifiers to collect original papers or relevant unpublished documents and to review them for GHS classification. Therefore, in general, international or national review documents or databases should be used as data sources for mutagenicity test results for the classification (NITE, 2005a). The major 21 information sources used in J-GHS are from International Program on Chemical Safety (IPCS), European Union (EU), OECD, US, Germany, Canada, Australia and Japan. These information sources with their abbreviated names or web addresses are shown in Table 2. Most of them can be accessed easily on the internet free of charge. Some original papers were also used for the review of the classification results, if necessary.

The availability of suitably independent, commercial, in confidence, data and documents from industry sources (a major source of genotoxicity data) is also a problem.

4. Development of supportive guides for GHS classification

The Guidance for practical classification of GCM has been prepared by J-GHS. It consists of precise explanations of GCM for GHS classification, additional examples of mutagenicity or genotoxicity tests, and a practical decision tree for classification of GCM. Definition of criteria of germ cell mutagens in GHS are also proposed here. These practical approaches will be of help to Classifiers.

4.1. Additional examples of mutagenicity or genotoxicity tests for classification

In the GHS (UN, 2007), the term "mutation" applies both to heritable genetic changes that may be manifested at the phenotypic level and to the underlying DNA modifications when known (including, for example, specific base pair changes and chromosomal translocations). The terms "mutagenic" and "mutagen" are used for chemicals giving rise to an increased occurrence of mutations in populations of cells and/or organisms (UN, 2007). The more general terms "genotoxic" and "genotoxicity" apply to chemicals or processes which alter the structure, information content, or segregation of DNA, including those which cause DNA damage by interfering with normal replication processes, or which in a non-physiological manner (temporarily) alter its replication. GHS pro-

Table 2
Major information sources used for GHS classification for germ cell mutagens in the Japanese GHS Classification Project (J-GHS).

Abbreviated name	Information source	Access or note
ACGIH	Documentation of the threshold limit values for chemical substances by American Conference of Governmental Industrial Hygienists	7th edition (2001 or later) Issued by ACGIH (http://www.acgih.org/home.htm)
ATSDR	Toxicological Profile by US Agency for Toxic Substances and Disease Registry	http://www.atsdr.cdc.gov/toxpro2.html
CERI	Chemical hazard data sheet by Chemicals Evaluation and Research Institute	In Japanese http://www.cerij.or.jp/db/date_sheet_list/list_sideindex_cot.html
CERI-NITE	CERI-NITE hazard assessment report by Chemicals Evaluation and Research Institute (CERI) and National Institute of Technology and Evaluation (NITE)	In Japanese http://www.safe.nite.go.jp/data/sougou/pk_list.html?table_name=hyoka_risk
CICAD	Concise International Chemical Assessment Document by International Program on Chemical Safety (IPCS)	http://www.inchem.org/pages/cicads.html
DFGOT	Occupational Toxicants: Critical Data Evaluation for MAK Values and Classification of Carcinogens by Deutsche Forschungsgemeinschaft (DFG, German Research Foundation)	http://www.dfg.de/en/ Issued by WILEY-VCH (The MAK-Collection, http://www.wiley-vch.de/books/info/mak/collection.php)
ECETOC	Technical Report by European Center of Ecotoxicology and Toxicology of Chemicals	http://www.ecetoc.org/publications
EHC	Environmental Health Criteria by IPCS	http://www.inchem.org/pages/ehc.html
EURAR	European Union Risk Assessment Report by European Chemical Bureau (ECB)	http://ecb.jrc.it/home.php?CONTENU=/DOCUMENTS/Existing-Chemicals/RISK_ASSESSMENT/REPORT/
HSDB	Hazardous Substance Data Bank by US National Library of Medicine, Toxicology Data Network	http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB
IARC	Monographs on the Evaluation of Carcinogenic Risk to Humans by International Agency for Research on Cancer	http://monographs.iarc.fr/ENG/Monographs/allmonos90.php and Printed versions
IRIS	Integrated Risk Information System by US Environmental Protection Agency	http://cfpub.epa.gov/ncea/iris/index.cfm
IUCLID	International Uniform Chemical Information Database in European chemical Substances Information System by ECB	http://ecb.jrc.it/esis/esis.php?PGM=hpv&DEPUIS=autre
JECDB	Japan Existing Chemical Data Base by Ministry of Health, Labor and Welfare in Japan, Toxicity Testing Report for Environmental Chemicals	In Japanese http://dra4.nihs.go.jp/mhlw_data/jsp/SearchPageENG.jsp
NITE	Preliminary risk evaluation report of chemicals by National Institute of Technology and Evaluation	In Japanese http://www.safe.nite.go.jp/risk/riskhykdl01.html
NTP DB	Testing Information Data Base by US National Toxicology Program	http://ntp.niehs.nih.gov:8080/index.html?col=010stat
PATTY	Patty's Toxicology by Bingham, E., Cohrssen, B., Powell, C.H., (eds.)	5th edition (2001) Issued by John Wiley and Sons (http://www3.interscience.wiley.com/cgi-bin/mrwhome/104554795/HOME?CRETRY=1&SRETRY=0)
PECAR	Priority Existing Chemical Assessment Reports in National Industrial Chemical Notification and Assessment Scheme by Australia	http://www.nicnas.gov.au/publications/car/PEC.asp
PSAR	Priority Substance Assessment Reports by Environment Canada	http://www.hc-sc.gc.ca/ewh-semt/contaminants/existsub/psap-pesip_e.html Full set by CD-ROM
RTECS	Registry of Toxic Effects of Chemical Substances by US National Institute for Occupational Safety and Health	http://www.cdc.gov/niosh/npg/npgdrtec.html or other commercial providers
SIDS	OECD Screening Information Data Set by United Nations Environmental Program	http://www.chem.unep.ch/irptc/sids/OECDSIDS/sidspub.html OR http://www.inchem.org/pages/sids.html

vides hazard categories and their criteria for germ cell mutagens including the examples of mutagenicity/genotoxicity tests to be used for classification (see Table 1). However, the examples presented in the GHS text are not sufficient for the classification. There are many kinds of mutagenicity or genotoxicity tests developed, some of which are validated or are being validated on the detection of mutagenic or genotoxic effects of chemicals. These include gene mutation tests with transgenic animal models, assays of (covalent) binding or adduct formation to DNA *in vivo*, assays of DNA damage *in vivo* including comet assay and *in vitro* chromosome damage assays such as the mammalian cell micronucleus test. Some of these tests are used in the EU (European Communities, 2001; Pratt and Barron, 2003), German Maximale Arbeitsplatz-Konzentration (MAK) Commission (Adler et al., 2000; DFG, 2007) or US EPA scientists (Dearfield et al., 2002) for the classification of mutagens (Morita et al., 2006). Human monitoring or epidemiological data (e.g., chromosome analysis of peripheral lymphocytes, comet assays in lymphocytes or sperm) will be also available and useful for certain chemicals, though these data might be insufficient for drawing general conclusions. Additional examples of mutagenicity/genotoxicity tests for practical GHS classification of GCM are shown in Table 3. On the other hand, several tests are considered not to be used for classification in general. These tests include a number of *in vitro* genotoxicity tests, e.g., the comet assay or UDS test using mammalian cultured cells, host-mediated assays using bacterial gene mutation systems, DNA-repair test (Rec-assay) in bacteria, umu test or SOS test using bacteria, gene conversion test or aneuploidy test using yeast. The sperm abnormality test using rodents and several *Drosophila* tests (sex-linked recessive lethal test, wing spot test, etc.) also are not used for classification in principle. This is because sperm abnormalities might be due sometimes to the effects on targets other than genetic material, and the ADME (absorption, distribution, metabolism, excretion) profile and reproductive and developmental processes in insects differ from those in mammals. However, these tests may be considered on a case-by-case basis with expert judgment.

4.2. Practical decision tree for classification of germ cell mutagens and proposed definition of them for GHS

GHS provides the decision logic for germ cell mutagenicity. Simple decision trees have been devised, because expert judgment on a weight of evidence approach is always needed for application of the GHS criteria (see Fig. 1). However, it is difficult for Classifiers who are not expert in this field to classify the chemicals without guidance if they use it in combination with criteria of hazard categories in Table 1. Therefore, a practical decision tree for classification of GCM in GHS has been developed (Fig. 2). The judgment in the practical decision tree flows from upstream (i.e., Category 1A) to downstream (i.e., Category 2 or Not yet classified as genotoxic [as Not classified]) as well as the original tree in GHS. The practical tree uses all tests to be used for classification shown in Table 3. Each number in the box in Fig. 2 corresponds to the test examples in Table 3. The basic concept of this tree is that positive results outweigh negative results in each test because, when conflicting results were obtained, negative results sometimes arise from

Table 3
Examples of mutagenicity or genotoxicity tests for practical GHS classification of germ cell mutagens.

#1	*In vivo* heritable germ cell mutagenicity tests in mammals
	1.1 Mouse specific locus test
	1.2 Mouse heritable translocation test
	1.3 Rodent dominant lethal test
#2	*In vivo* germ cell mutagenicity tests in mammals
	2.1 Chromosomal aberration test in spermatogonia
	2.2 Micronucleus test in spermatid cells
	2.3 Gene mutation test in germ cells of transgenic rodents*
	2.4 Analysis of aneuploidy in sperm cells of exposed people*
#3	*In vivo* somatic cell mutagenicity tests in mammals
	3.1 Chromosome aberration test in bone marrow cells or peripheral lymphocytes
	3.2 Mouse spot test
	3.3 Micronucleus test in hematopoietic cells
	3.4 Gene mutation test in somatic cells of transgenic rodents*
	3.5 Metaphase or micronucleus formation analysis of peripheral lymphocytes of exposed people*
#4	*In vivo* germ cell genotoxicity tests in mammals
	4.1 Sister chromatid exchange (SCE) test in spermatogonia
	4.2 Unscheduled DNA synthesis (UDS) test in testicular cells
	4.3 Assay of covalent binding or adduct formation to germ cell DNA*
	4.4 Assay of DNA damage in germ cells (comet assay, alkaline elution assay, etc.,)*
#5	*In vivo* somatic cell genotoxicity tests in mammals
	5.1 UDS test in liver
	5.2 SCE test in bone marrow cells or peripheral lymphocytes
	5.3 Assay of covalent binding or adduct formation to somatic cell DNA*
	5.4 Assay of DNA damage in somatic cells (comet assay, alkaline elution assay, etc.,)*
	5.5 SCE analysis of peripheral lymphocytes of exposed people*
#6	*In vitro* mutagenicity tests
	6.1 Chromosomal aberration test in cultured mammalian cells
	6.2 Micronucleus test in cultured mammalian cells*
	6.3 Gene mutation test in cultured mammalian cells
	6.4 Reverse mutation test in bacteria (i.e., Ames test)

* Added to the examples in GHS.

inadequate experiments. Therefore, judgment of accuracy of the negative results, especially in table form, will be difficult for Classifiers using the GHS classification. While a single positive result can sometimes be pivotal to a decision about a classification, such findings should be considered on a case-by-case basis and are not necessarily definitive for classification.

Criteria of practical GHS classification of GCM used in the present J-GHS is presented in Table 4. This will be helpful to understand the practical classification scheme suggested. When human heritable germ cell mutagenicity is identified by human epidemiological studies, the substance will be classified as Category 1A, and can be regarded as a "human heritable germ cell mutagen" (Table 4). The existence of any such substance is not confirmed at present. However, exposures in the environment are far too many and far too complex, as are the potential genetic targets. Therefore, this does not mean that such substances do not exist.

When a positive result(s) from an *in vivo* mutagenicity test is available with suggestive data on mutagenicity in germ cells, the substance will be classified as Category 1B. Practically, the following examples will apply for this category: (1) positive results in heritable germ cell mutagenicity tests in mammals, e.g., dominant lethal test, heritable translocation test, or specific locus test; (2) positive results from *in vivo* germ cell mutagenicity tests in mammals, e.g., chromosomal aberration test in mammalian spermatogonia, micronucleus test in mammalian spermatid cells, or gene mutation test in germ cells of transgenic rodents (preferably, this will be supported by the positive result(s) from *in vivo* somatic cell mutagenicity tests in mammals); (3) positive findings of mutagenicity in human germ cells with no evidence of transmission to progeny, e.g., an increase in the frequency of aneuploidy in sperm cells of exposed people; (4) positive results from *in vivo* somatic cell mutagenicity test in mammals, e.g., chromosomal aberration test in mammalian bone marrow cells or peripheral lymphocytes, micronucleus test in mammalian hematopoietic cells, gene mutation test in somatic cells of transgenic rodents, or mouse spot test, with positive result(s) from *in vivo* germ cell genotoxicity tests in mammals, e.g., sister chromatid exchanges (SCE) test in mammalian spermatogonia, unscheduled DNA synthesis (UDS) test in mammalian testicular cells, assay of covalent biding or adduct formation to mammalian germ cell DNA, or assay of DNA damage (comet assay, alkaline elution assay, etc.) in mammalian germ cells, and; (5) positive results from *in vivo* somatic cell mutagenicity tests in mammals, with demonstration of exposure of the substance or its metabolite(s) to germ cells (preferably, with relevant route of exposure). A substance classified as Category 1B can be regarded as a "mammalian germ cell mutagen" (Table 4). Category 1B is similar to the categories of probable human germ cell mutagen and possible human germ cell mutagen in the proposed classification categories of Dearfield et al. (2002).

When positive result(s) from any *in vivo* somatic cell mutagenicity/genotoxicity test in mammals is available without supportive evidence of the mutagenicity in germ cell, the substance will be classified Category 2. The following cases will be normally classified in this category: (1) positive result(s) from *in vivo* somatic cell mutagenicity tests in mammals (see above); (2) positive finding(s) of mutagenicity in human somatic cells, e.g., an increase in the frequency of chromosomal aberrations or micronuclei in peripheral lymphocytes of exposed people; (3) positive result(s) in germ or somatic cell genotoxicity tests in mammals, e.g., SCE test in mammalian bone marrow cells or peripheral lymphocytes, UDS test in mammalian liver, assay of covalent biding or adduct formation to mammalian somatic cell DNA, or assay of DNA damage (comet assay, alkaline elution assay, etc.) in mammalian somatic cells, with positive result(s) from *in vitro* mutagenicity tests, i.e., chromosomal aberration test, micronucleus test, or gene mutation test in cultured mammalian cells, or reverse mutation test in bacteria; (4) positive finding(s) of genotoxicity in human somatic cells, e.g., an increase in the frequency of SCE in peripheral lymphocytes of exposed people, with positive result(s) from *in vitro* mutagenicity tests, and; (5) as a special case, (strong) positive results from *in vitro* mutagenicity tests with at least two different endpoints (without *in vivo* mutagenicity/genotoxicity test data), with a chemical structure activity relationship to known germ cell mutagens (Category 1 or 2 substances). In this case, expert judgment will be required. The "mutagen" covers the chemicals which induce the three levels of genetic damage, i.e., mutagenicity (gene mutation), clastogenicity (structural chromosomal aberration) and aneuploidy (numerical chromosomal aberration). The evidence that supports clastogenicity *in vivo* but not mutagenicity will lead to Category 1 or 2. However, if the only evidence of clastogenicity is from *in vitro* studies, this is insufficient for Category 1 or 2, without supportive data. In all categories, it is preferable for *in vivo* positive result(s) to be supported by the positive result(s) *in vitro*. This category can be regarded as describing "mammalian somatic cell mutagen" (Table 4).

When a negative result(s) in any *in vivo* germ or somatic cell mutagenicity tests is available, the substance will be classified "Not yet classified as genotoxic [as Not classified]". Substances with limited evidence (not sufficient evidence) in somatic cell mutagenicity/genotoxicity tests might be also considered as "Not yet classified as genotoxic". This situation might include negative result(s) from *in vivo* somatic and germ cell mutagenicity tests, but positive result(s) from *in vitro* mutagenicity tests. Expert judgment in a weight of evidence approach will be important to give a final call of classification in this example. This category can be regarded as "Not likely to be mammalian mutagen" (Table 4).

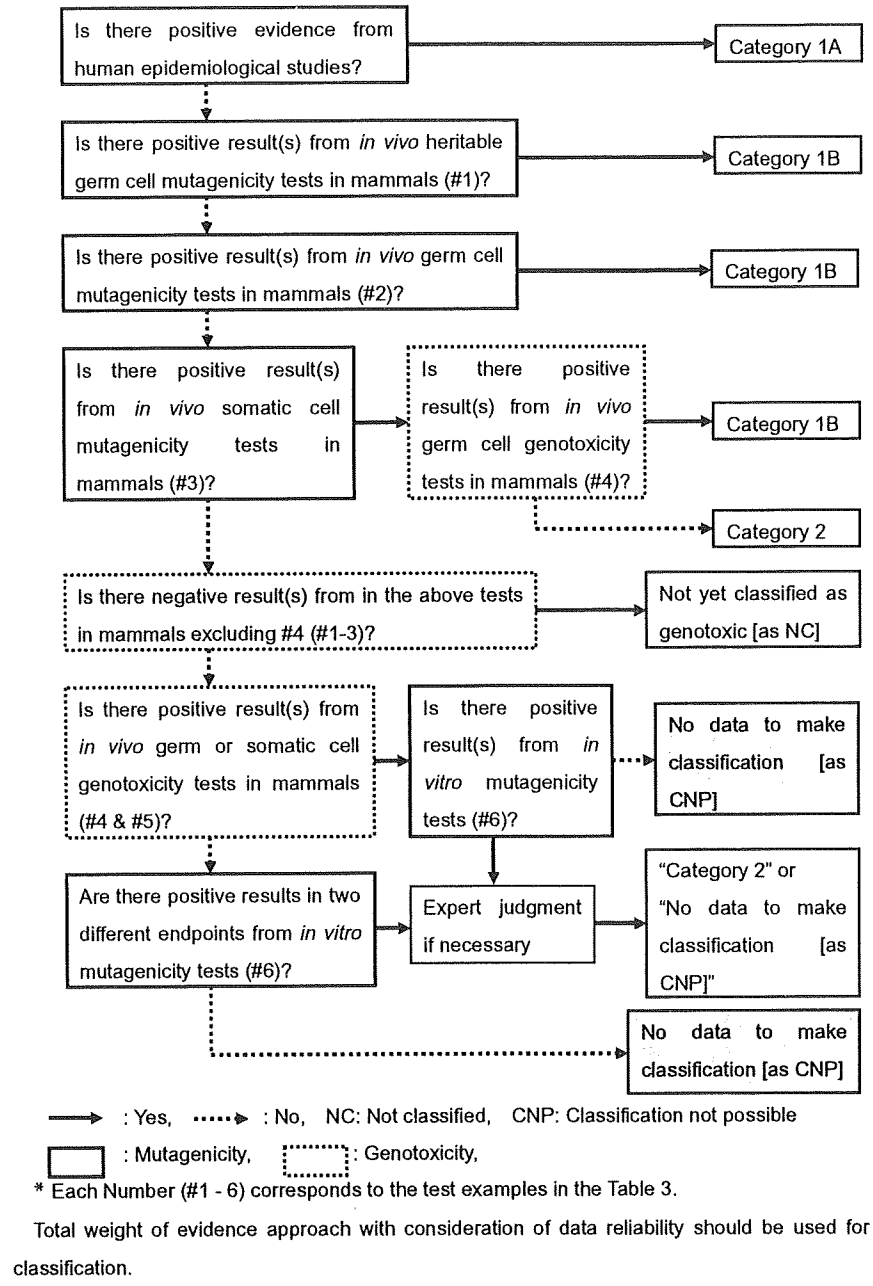

Fig. 2. Practical decision tree for classification of germ cell mutagens for GHS. Solid line is for Yes, dotted line is for No. Bold solid line box is for mutagenicity test set, dotted line box is for genotoxicity test set. Each number (#1–6) corresponds to the test examples in Table 3. Total weight of evidence approach with consideration of data reliability should be used for classification.

When no data from *in vivo* mutagenicity or genotoxicity tests in mammals are available, the substance will be classified "No data to make classification [as Classification not possible]". In addition, the case of no data from *in vivo* mutagenicity tests AND positive result(s) from *in vivo* somatic cell genotoxicity tests AND negative result(s) in any *in vitro* mutagenicity tests gives "No data to make classification". A recent analysis demonstrated that there is an extremely high false positive rate for *in vitro* mutagenicity/genotoxicity tests, when compared with carcinogenicity in rodents (Kirkland et al., 2005). It implies that reliable heritable genetic risk determination as well as cancer health risk cannot be made the basis of *in vitro* findings alone (Thybaud et al., 2007b). Exposure to germ cells should also be considered. Therefore, it is difficult to estimate human heritable germ cell mutagenicity from only the results of *in vitro* mutagenicity tests. When positive result(s) from only *in vitro* mutagenicity test data are available, the substance is also classified "No data to make classification" in principle. An exceptional case is described above (5) as a special case in Category 2.

5. Examples of results on the selected chemicals and re-evaluation

About 1400 chemicals regulated by Japanese laws were submitted for classification. Data for evaluation were obtained from selected documents or databases (see Section 3). Classification was performed by non-experts based on the classification guid-

Table 4
Criteria of practical GHS classification of germ cell mutagens.

GCM	GHS category	Practical classification [explanation]	Criteria [test*]
GCM	Category 1A	Human heritable germ cell mutagen [Human germ cell mutagen]	(1) Positive evidence from human epidemiological studies (no compound identified so far)
	Category 1B	Mammalian germ cell mutagen [Probable human germ cell mutagen]	(1) Positive result(s) from *in vivo* heritable germ cell mutagenicity tests in mammals [#1] (2) Positive result(s) from *in vivo* germ cell mutagenicity tests in mammals [#2] (preferably, this will be supported by the positive result(s) from *in vivo* somatic cell mutagenicity tests in mammals) (3) Positive findings from analysis showing mutagenic effects in the germ cells of humans, without demonstration of transmission to progeny [#2.4] (4) Positive result(s) from *in vivo* somatic cell mutagenicity tests in mammals AND positive result(s) from *in vivo* germ cell genotoxicity tests in mammals [#3 + #4] (5) Positive result(s) from *in vivo* somatic cell mutagenicity tests in mammals AND demonstration of exposure to germ cells by substance or active metabolite(s) [#3 + E] (preferably, in relevant route of exposure)
	Category 2	Mammalian somatic cell mutagen [Possible human germ cell mutagen]	(1) Positive result(s) from *in vivo* somatic cell mutagenicity tests in mammals [#3] (2) Positive results from tests showing mutagenic effects in the somatic cells of humans [#3.5] (3) Positive result(s) from *in vivo* germ or somatic cell genotoxicity tests in mammals AND positive result(s) from *in vitro* mutagenicity tests [(#4 or #5) + #6] (4) Positive finding(s) from analysis showing genotoxic effects in the somatic cells of humans AND positive result(s) from *in vitro* mutagenicity tests [#5.5 + #6] (5) (Strong) positive result(s) from *in vitro* mutagenicity test(s) with at least two different end points AND showing chemical structure activity relationship to known germ cell mutagens (i.e., Category 1or 2 substances) as special case [#6 + C]
Non-GCM	Not classified (Not yet classified as genotoxic)	Not likely to be mammalian mutagen [Probable non human germ cell mutagen]	(1) Negative result(s) in any *in vivo* germ or somatic cell mutagenicity tests in mammals (2) No sufficient evidence in somatic cell mutagenicity tests in mammals
–	Classification not possible (No data to make classification)	–	(1) No data on *in vivo* mutagenicity or genotoxicity tests in mammals (2) No data on *in vivoi* mutagenicity tests in mammals AND positive result(s) from *in vivo* genotoxicity tests in mammals AND negative result(s) in any *in vitroi* mutagenicity test(s) (3) Data available only *in vitro* mutagenicity test(s) except for special case (see criteria 5) in Category 2)

* Each number is corresponding to the test number in Table 3, "E" means exposure of substance or its metabolite(s) to germ cells, and "C" means chemical structure activity relationship to known germ cell mutagens.

ance and the practical decision tree. Expert review on the classification was limited to those chemicals that were considered to need the help of experts. Therefore, some of the results in this project will not be suitable for reliable classification: about 3% and 13% of chemicals were in Category 1B and Category 2, respectively. Nearly 30% of chemicals were in "Not Classified (NC)". More than 50% of chemicals were "Classification Not Possible (CNP)", due to no or insufficient data for classification. The high percentage of CNP's reflects the chemicals used in this project that are regulated by specific laws (e.g., the Industrial Safety and Health Law, the Poisonous and Deleterious Substances Control Law, and PRTR) in Japan.

The results of the classification on 20 chemicals are shown in Table 5. When questionable classification results were recognized by experts, re-evaluation was performed based on the new data search, evaluation of data reliability, and weight of evidence approach. The selected chemicals included ones that required re-evaluation by experts (acrylonitrile, phenol, nitrilotriacetic acid, ethanol, sodium chlorite and 1-chloro-2-nitrobenzene), or that were representative examples for classification using the practical decision tree (the other chemicals).

5.1. Acrylamide [79-06-1] (J-GHS ID 0001), Cat. 1B

Many positive results were found in variety of genotoxic endpoints including heritable mutagenicity (CERI-NITE, 2004b). Therefore, GHS Category 1B was applied. This is supported by EU Mutagenicity Category 2 in the EU Annex I (ECB, 2008) and MAK Germ Cell Mutagenicity (GCM) Category 2 in the MAK List (DFG, 2007).

5.2. Acrylic acid [79-10-7] (J-GHS ID 0002), NC

Negative results were obtained from a dominant lethal test and a chromosomal aberration test using rodents (ECB, 2002c), although positive results were obtained from an *in vitro* chromosomal aberration test and a gene mutation test with mammalian cells. Based on these findings, "Not Classified (NC)" was assigned according to the practical GHS decision tree. Acrylic acid is not categorized as a mutagen or GCM in EU Annex I or the MAK List, respectively.

5.3. Acrylonitrile [107-13-1] (J-GHS ID 0003), Changed to NC from Cat. 2

One positive finding was reported from *in vivo* mutagenicity test (rat splenic T-cell *hprt* mutation assay) together with one positive finding from *in vivo* genotoxicity test (liver UDS assay). In addition, *in vitro* mutagenicity tests in mammalian cells were also positive. On the other hand, negative results were obtained in rodent dominant lethal tests, mouse spermatogonial chromosomal aberration test and rat spermatocyte UDS tests (IPCS, 2002; CERI-NITE, 2003; ECB, 2004a). Following the practical decision tree,